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Cloning and characterization of an isoform of interleukin-21

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Abstract Interleukin-21 (IL-21) has pleiotropic functions on the cells, which play roles in both innate and acquired immunity, such as T cells, B cells, natural killer (NK) cells and dendritic cells. In this study we identified a novel isoform of IL-21, IL-21iso in human and mouse. IL-21iso might be an alternative splicing variant form and the C-terminal region of predicted IL-21iso amino acid sequences were different from original IL-21 in both human and mouse. In spite of the differences in C-terminal amino acid sequences, both human IL-21 and IL-21iso showed comparable proliferative effect on anti-CD40 Ab-activated primary B cells, anti-CD3 Ab-activated primary T cells and human NK cell line, NK0, and upregulated IFN-γ production from NK0. Furthermore IL-21 and IL-21iso similarly activated STAT1 and STAT3. IL-21iso mRNA was expressed in activated T cells as well as IL-21 mRNA. However, cycloheximide treatment partially blocked the upregulation of IL-21iso mRNA in activated T cells while little affected the IL-21 mRNA expression suggesting that *de novo* protein synthesis is required for the full expression of IL-21iso transcript. We also show that the secretion efficiency of hIL-21iso is much lower than that of hIL-21. These results may suggest there are some different regulatory mechanisms to produce IL-21 or IL-21iso in transcriptional and secretory steps.

Keywords: Interleukin-21; Isoform; Cytokine

1. Introduction

Interleukin-21 (IL-21) is a T cell-derived cytokine, which was discovered in 2000 with its receptor (IL-21R) [1,2]. IL-21 is most similar to IL-2, IL-4 and IL-15 whereas IL-21R has structural similarity with IL-2Rβ and IL-4Rα. IL-21 is a member of the common γ chain (γc)-dependent cytokine family that includes IL-2, IL-4, IL-7, IL-9 and IL-15 [3,4]. Because X-linked severe combined immunodeficiency (X-SCID) is caused by γc mutations in a gene on the X chromosome [5], it was expected that IL-21 dysfunction had important roles in the pathogenesis of X-SCID. In fact, IL-21R/IL-4 double-deficient mice showed B cell dysfunction as X-SCID patients [6].

IL-21R is preferentially expressed on B cells, T cells, natural killer (NK) cells, some myeloid cells, and keratinocytes [1,7,8]. Therefore, IL-21 has diverse functions on various immune cells of innate and adaptive immunity [1,9]. IL-21 costimulates the proliferation of activated T cells and induces the differentiation of B cells into memory cells and plasma cells [1,10]. Interestingly, IL-21 suppresses IgE production through inhibition of germ line Cε transcription and Bε cell apoptosis [11,12]. On NK cells different biologic effects of IL-21 between human and mouse are reported. Human IL-21 (hIL-21) enhances NK cell cytotoxic activity and NK cell proliferation while mouse IL-21 (mIL-21) enhances NK activity but inhibits NK cell proliferation [1,9,13,14]. IL-21 inhibits dendritic cell activation and maturation [15] and lipopolysaccharide-induced cytokine production in human monocyte-derived dendritic cells [16]. Moreover, IL-21 might have a role in the development of autoimmunity [10,17,18] and many reports show its anti-tumor activities [19] suggesting that the function of hIL-21 is closely related in human diseases including tumor development.

During an analysis of IL-21 transcripts with RT-PCR method, we found additional products in human and mouse. We have cloned them and DNA sequence analysis showed that the clones were IL-21isoforms, IL-21iso. Among the γc-dependent cytokine family, human IL-2 and human IL-4 isoforms were reported to be competitive inhibitors to the original IL-2 and IL-4, respectively [20,21]. On the other hand, human IL-21iso (hIL-21iso) has comparable function with hIL-21 on the proliferation of anti-CD40 Ab-activated B cells, anti-CD3 Ab-activated T cells and human NK cell line, NK0 cells and on the IFN-γ production from NK0 cells. We also show that IL-21 and IL-21iso are differentially regulated in the transcriptional and cytokine secretory steps.

2. Materials and methods

2.1. Cells and antibodies

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood of healthy donors by density gradient centrifugation using Ficoll-Paque™ Plus (Amersham Pharmacia Biotech., London, UK) according to the manufacturer's instructions. Isolated PBMC were maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS, penicillin and streptomycin. B cells were purified from PBMC with anti-CD19 magnetic beads after depleting monocytes with anti-CD14 magnetic beads (BD Biosciences Pharmingen, San Diego, CA). To purify $CD4^+$ T cells, both $CD14^+$ cells and

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Abbreviations: Ab, antibody; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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$CD19^+$ cells-depleted PBMC was further purified with anti-CD4 magnetic beads (BD Biosciences Pharmingen). Human NK cell line NK0 cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 1 nM recombinant human IL-2 (rhIL-2, R&D Systems, Minneapolis, MN, USA), 100 mM sodium pyruvate, MEM non-essential amino acids, penicillin and streptomycin [22]. Ba/F3 is an IL-3-dependent murine pro-B cell line and the subline, BAF3-6 cell, lacks IL-21R expression [23]. We prepared human IL-21R (hIL-21R) cDNA from total RNA of human peripheral blood B cells and cloned into pEFneo expression vector plasmid [23]. BAF21RWT-1 cell line was established by introducing hIL-21R expression vector plasmid, pEFneohIL-21R, into BAF3-6 cells. The expression level of hIL-21R on the BAF21RWT-1 cell was determined by flow cytometry with anti-hIL-21R monoclonal antibody (mAb) (clone 17A12, unpublished data). BAF3-6 and BAF21RWT-1 cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 15% conditioned medium of WEHI3 cells as a source of IL-3, penicillin and streptomycin. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, penicillin and streptomycin. Mouse splenocytes from ICR mice aged 8–12 weeks were maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS and 50 μM 2-mercaptoethanol. These cells were cultured at 37 °C in a humidified incubator with 5% CO_2. Anti-human CD3 mAb (UCHT1) and FITC-labeled anti-human IFN-γ mAb were purchased from BD Biosciences Pharmingen. Anti-human CD40 mAb (S2C6) was purchased from Mabtech AB. Anti-phospho-STAT1 (Y701) Ab, anti-phospho-STAT3 (Y705) Ab and HRP-conjugated anti-rabbit IgG Ab were purchased from Cell Signaling Technology (Beverly, MA) and anti-STAT1 Ab and anti-STAT3 Ab were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). We prepared anti-hIL-21 mAb (4BG1) and anti-hIL-21 polyclonal Ab by ourselves (manuscript in submission). All human specimens were obtained under informed consent. The protocol for the human research has been approved by the Ethics Committee of Yamagata University Faculty of Medicine. All mouse experiments were performed in accordance with the guidelines of the Laboratory Animal Center of Yamagata University Faculty of Medicine and were approved by the animal experiment committee of Yamagata University Faculty of Medicine.

2.2. RNA extraction and RT-PCR

Human $CD4^+$ T cells or mouse spleen cells were treated with or without cycloheximide (Sigma, St. Louis, MO), phorbol 12-myristate 13-acetate (PMA) (Sigma) or ionomycin (Sigma) as indicated. Total cellular RNA was isolated from these cells using TRIzol™ Reagent (Invitrogen, San Diego, CA). First strand cDNA was synthesized from 1 μg of total RNA using AMV Reverse Transcriptase and oligo (dT) primer with cDNA synthesis kit (Life Sciences, Inc., St. Petersburg, FL) according to the manufacturer's instructions. The synthesized first-strand cDNAs were amplified by PCR using primers for IL-21, IL-21iso, IL-2 or β-actin with *TaKaRa Taq*™ *HS* (TaKaRa Bio Inc. Kyoto, Japan). Primers used in this study are described as follows, for total hIL-21: hF1 5′-AAGGTACCACTTATGAGATCCAGTCCT-3′ and hR1 5′-AAACTAGTCAACTGCAAGTTAGATCCT-3′, specific for hIL-21iso: hF2 5′-CCAACTAAAGTCAGCAAATACAGG-3′ and hR2 5′-GTGAGTAACTAAGAAGCAAATCTGG-3′, specific for hIL21: hF3 5′-CACTTCTCCAAAAGATGAT-3′ and hR3 5′-ATCCTGACTTTGCACACT-3′, for

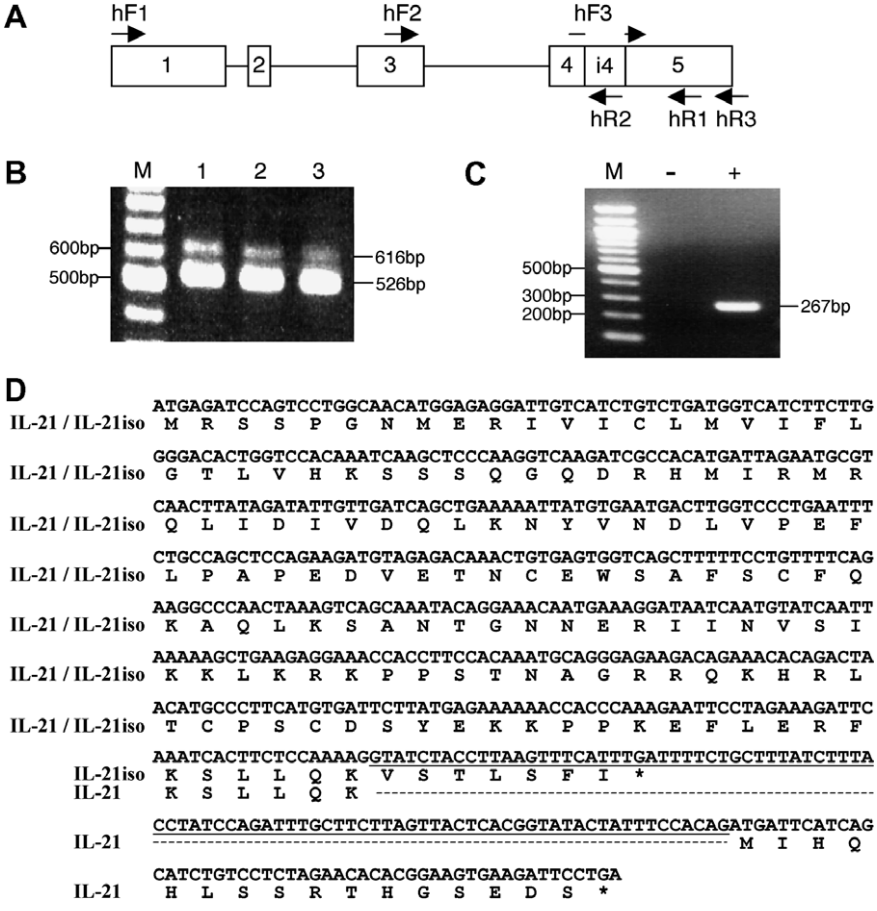

Fig. 1. Cloning of a novel IL-21 isoform in human. (A) Schematic structure of hIL-21 gene. Exons are indicated as boxes with numbers and intron 4 is indicated as i4. Positions of primers used for PCR are shown as arrows. (B) cDNAs prepared from anti-human CD3 mAb activated PBMC from 3 healthy donors were amplified by PCR using hF1 and hR1 primers. M indicates 100 bp DNA ladder marker. (C) cDNAs prepared from $CD4^+$ T cells before (−) and after activation with anti-human CD3 mAb (+) for 6 h were amplified by PCR using hF2 and hR2 primers. (D) Sequence comparison between hIL-21 and hIL-21iso. Additional DNA sequences of hIL-21iso are underlined. Asterisk (*) indicates the conceptual stop codon.

human IL-2 (hIL-2): 5′-GTTGTTTCAGATCCCTTTAGTTCCA-3′ and 5′-ACAGAACTGAAACATCTTCAGTGTC-3′, for human-β-actin: hβ-actinF 5′-CGACAGGATGCAGAAGGAGATCAC-3′ and hβ-actinR 5′-CGTCATACTCCTGCTTGCTGATCC-3′, for total mIL-21: mF1 5′-AAGGTACCATGGAGAGGACCCTTGT-3′ and mR1 5′-AAACTAGTATGTACTCCTGCATTCGTG-3′, specific for mIL-21iso: mF2 5′-GAGGAAAGAAACAGAAGCACA-3′ and mR2 5′-AGACACAACATGGAAGTGAAA-3′, specific for mIL-21: mF3 5′-TGGCTCCTTCAAAAGATGAT-3′ and mR1, for mouse IL-2 (mIL-2): 5′-GTTGTAAAACTAAAGGGCTCTGAC-3′ and 5′-GTTGAGATGATGCTTTGACAGAAG-3′, for mouse β-actin: 5′-TGACAGGATGCAGAAGGAGA-3′ and 5′-GCTGGAAGGTGG-ACAGTGAG-3′. Underlined sequences are restriction enzyme recognition sites for the DNA cloning in expression plasmid vectors.

Real time quantitative PCR amplifications were conducted using SYBR Green Realtime PCR Master Mix (Toyobo Co. Ltd. Osaka, Japan) according to the manufacturer's instructions, with 0.2 μM primers and cDNAs or a variable amount of DNA standard (pEFneo-hIL-21, pEFneohIL-21iso and pBShβactin for hIL21, hIL-21iso, and human β-actin, respectively) in a 20μl final reaction volume. Thermocycling was performed using a LightCycler (Roche Molecular System Inc. Pleasanton, CA) initiated by a 30 s incubation at 95 °C, followed by 40 cycles (95 °C, 5 s; 50 °C, 5 s; 72 °C, 20 s) with a single fluorescent reading taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. C_t values were determined using a fluorescence threshold set manually for all runs and exported into a MS Excel workbook (Microsoft Inc.) for analysis. hIL-21 or hIL-21iso mRNA expression level was normalized by dividing its relative mRNA amount with the corresponding β-actin mRNA amount. Primers used in the real time quantitative PCR are as follows, for hIL-21: hF3 and hR1, for hIL-21iso: hF2 and hIL-21isoSpeR 5′-AAACTAGTAAAGA-TAAAGCAGAAAAT-3′, for human β-actin: hβ-actinF and hβ-actinR.

2.3. IL-21 isoform cloning and construction of expression plasmid

Extra bands in human and mouse IL-21 PCR products were purified using hF1/hR1 or mF1/mR1 primers and digested with restriction enzymes, *KpnI* and *SpeI*. The digested fragments were ligated to pEFneo mammalian expression vector [23] and the nucleotide sequences were analyzed by ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

2.4. Cytokine production and quantification

293T cells were transfected with the hIL-21 or hIL-21iso expression vectors pEFneohIL-21 or pEFneohIL-21iso, respectively, or an empty vector, pEFneo, as a control by calcium-phosphate transfection method as previously described [24]. The supernatants were collected 48 h after transfection. The concentration of the hIL-21 and hIL-21iso in the supernatants was measured by immunoblot assay using anti-hIL-21 polyclonal Ab. These supernatants were used to stimulate cells in this study.

2.5. Cell proliferation assays

BAF3-6 and BAF21RWT-1 cells were cultured in 96-well flat-bottomed plates in triplicate with either hIL-21, hIL-21iso or recombinant mouse IL-3 (rmIL-3, R&D Systems, Minneapolis, MN, USA)

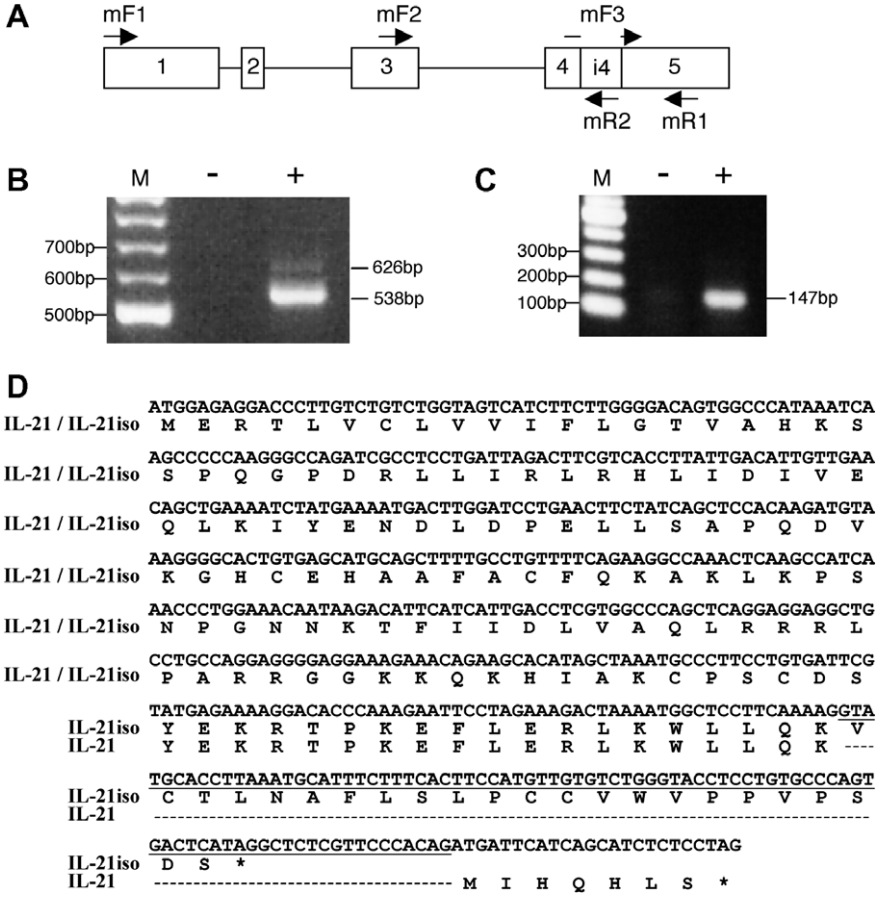

Fig. 2. Cloning of a novel IL-21 isoform in mouse. (A) Schematic structure of mIL-21 gene. Exons are indicated as boxes with numbers and intron 4 is indicated as i4. Positions of primers used for PCR are shown as arrows. Mouse spleen cells were cultured with (+) or without (−) PMA and ionomycin for 6 h. cDNAs prepared from these cells were amplified by PCR using mF1 and mR1 (B) or mF2 and mR2 (C). (D) Sequence comparison between mIL-21 and mIL-21iso. Additional sequences of mIL-21iso are underlined. Asterisk (*) indicates the conceptual stop codon.

for 24 h. Purified $CD19^+$ B cells stimulated with 1μg/ml anti-human CD40 mAb were cultured in 96-well flat-bottomed plates in triplicate with hIL-21, hIL-21iso or recombinant hIL-21 (rhIL-21, Biosource International, Camarillo, CA, USA) for 3 days. Purified $CD4^+$ T cells were cultured in triplicate with hIL-21 or hIL-21iso in 96-well flat-bottomed plates pre-coated with anti-human CD3 mAb (1 μg/ml), for 3 days. NK0 cells were washed three times with PBS and then cultured with or without hIL-21, hIL-21iso, rhIL-21 or rhIL-2 for 24 h. Then alamarBlue (BioSource Int. Inc. Camarillo, CA) was added to 10% of the total culture medium volume. Cells were cultured for an additional 24 h and absorbance was measured at 595 and 570 nm by plate reader. Because alamarBlue is more reduced in the proliferating cells than in the non-proliferating cells, cell proliferation was quantified as alamarBlue reduction. The percent alamarBlue reduced (%reduction) was calculated according to the manufacturer's instruction.

2.6. Functional assay on NK0 cells

To examine the functions of hIL-21 and hIL-21iso on human NK cell, we assessed the IFN-γ production from NK0 cells after hIL-21 or hIL-21iso treatment by intracellular staining with FITC-labeled anti-human IFN-γ antibody according to the manufacturer's instruction (BD Biosciences Pharmingen). Stained cells were analyzed by FACSCalibur (BD Biosciences, San Jose, CA).

2.7. Cell extraction and immunoblot assay

Cells were stimulated with hIL-21 or hIL-21iso at 1 nM concentration for 30 min. Cell extraction and immunoblot assay are described previously [23].

3. Results and discussion

3.1. Cloning of a novel IL-21 isoform

IL-21 is exclusively expressed from activated $CD4^+$ T cells. In order to examine the expression level of hIL-21 mRNA, we employed RT-PCR with hF1 and hR1 primers, allowing amplification of the entire coding region (Fig. 1A). Total RNA of anti-CD3 mAb-stimulated human PBMC was subjected to RT-PCR analysis and the products of the appropriate size (526 bp) were observed as expected. In addition, however, a larger band was also consistently observed (Fig. 1B). Nested PCR of the larger band using two inside primers indicated that the additional product might be derived from a hIL-21 transcript (data not shown). The larger product was isolated and the DNA sequences were analyzed. Interestingly, it was identi-

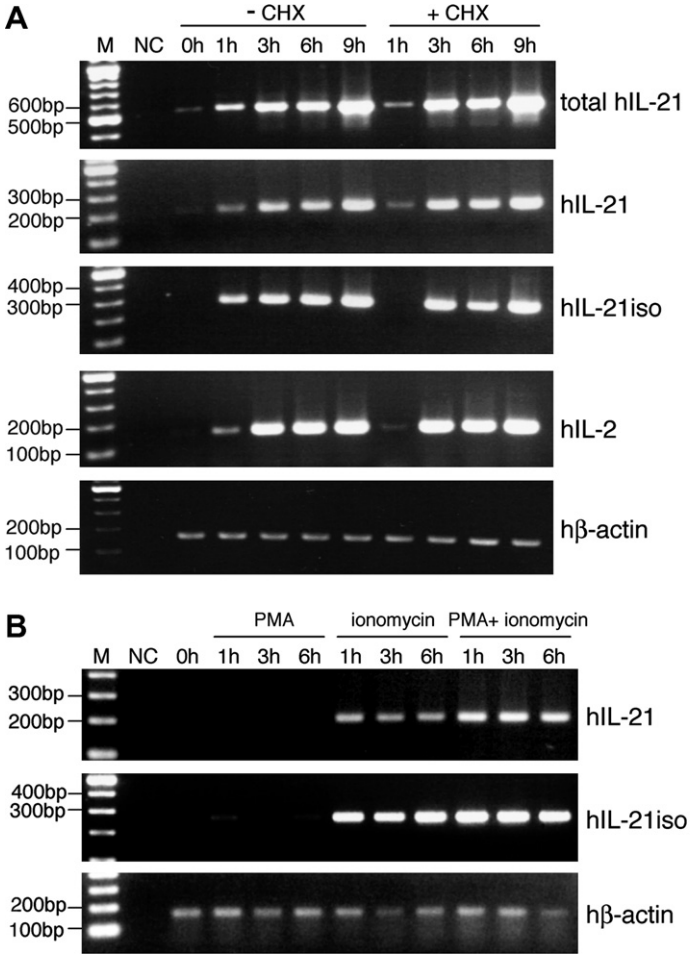

Fig. 3. Kinetics of IL-21 and IL-21iso mRNA expression. Human $CD4^+$ T cells or mouse spleen cells were treated with or without cycloheximide (10 μg/ml) for 1 h, and then stimulated with PMA (50 ng/ml) and ionomycin (1 μM) for the indicated periods (A and E). Human $CD4^+$ T cells or mouse spleen cells were also treated with PMA (50 ng/ml) or ionomycin (1 μM) separately as indicated (B and F). cDNAs prepared from these cells were used as templates for each PCRs. M and NC indicate 100 bp DNA ladder marker and negative controls without cDNA, respectively. Real time quantitative PCR experiments were performed as described in Section 2 with cDNA used in (A) and (B) (C and D). mRNA expression levels of hIL-21 and hIL-21iso were normalized with that of corresponding β-actin as indicated in Section 2. Representative results from at least 2 to 3 independent experiments are shown.

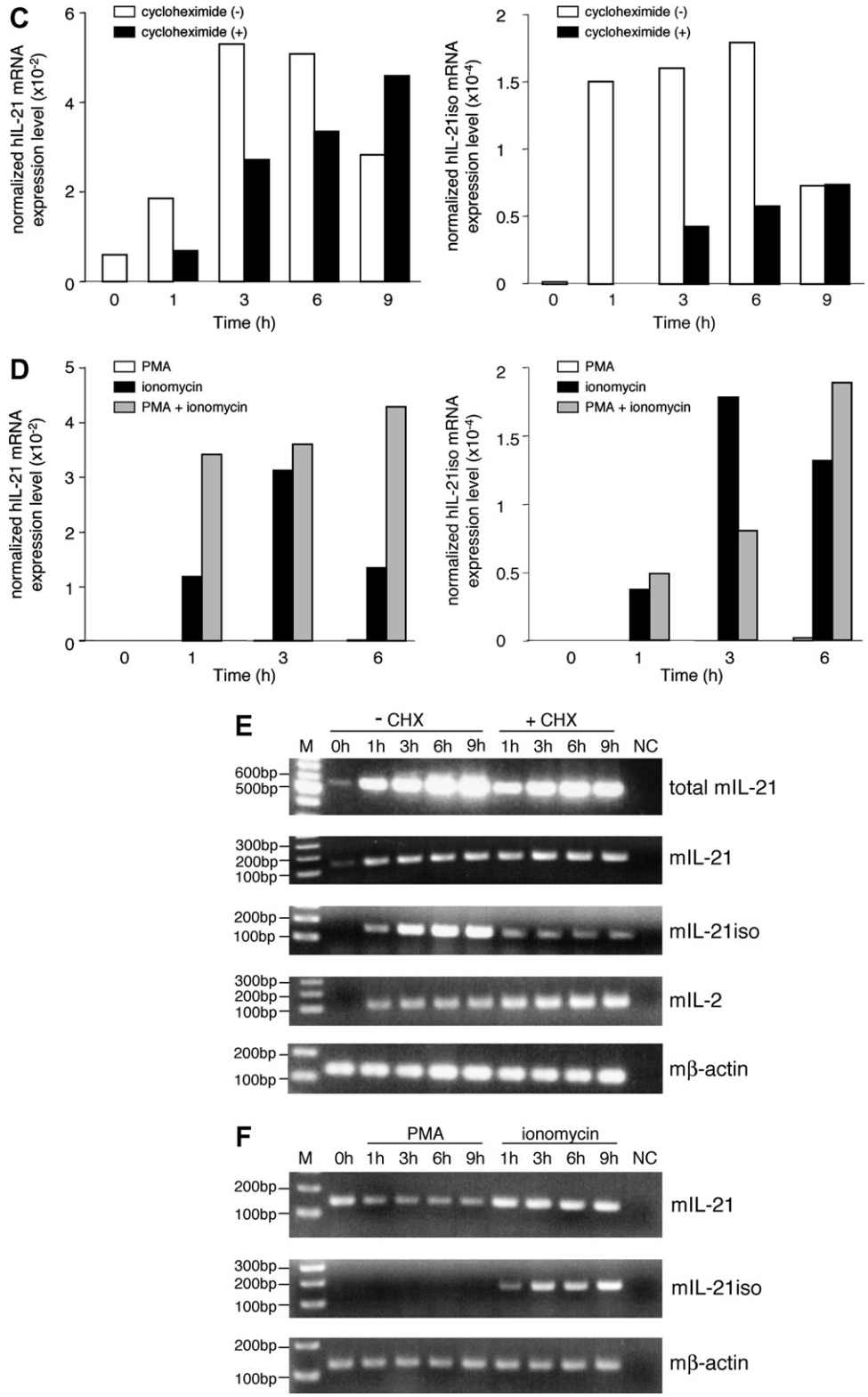

Fig. 3 *(continued)*

cal to hIL-21 cDNA sequence with an insertion of 90 bp unknown sequences. Data base search revealed that the 90 bp sequences are intron 4 sequences (i4) between exon 4 and exon 5 of already reported hIL-21 gene (Fig. 1A and D). We named this product hIL-21iso. This result indicates that mature mRNAs of hIL-21iso and hIL-21 are processed from the same pro-mRNA and hIL-21iso is an alternative splicing variant form. In the RT-PCR experiments we used oligo (dT) primer to prepare cDNA from total RNA indicating that only mature mRNAs might be amplified. Therefore we excluded a possibil-

ity that the isoform RNA was an intermediate in the middle of splicing. Since the additional 90 bp sequences include an in-frame stop codon, hIL-21iso is 9 amino acids shorter than the original hIL-21. So the predicted amino acid number of hIL-21iso precursor protein is 153 and mature hIL-21iso consists of 122 amino acids. To detect the hIL-21iso transcript clearly, we designed new primer set specific to hIL-21iso (hF2 and hR2) (Fig. 1A). $CD4^+$ T cells were isolated from PBMC and stimulated with or without anti-human CD3 mAb. RT-PCR using the IL-21iso specific primers demonstrated that hIL-21iso expression was found only in activated $CD4^+$ T cells (Fig. 1C).

Consequently, we hypothesized isoforms similar to hIL-21iso also exist in mouse. As expected, we found an additional larger band than 538 bp band in the amplified products from RT-PCR using mF1 and mR1 primers and cDNA prepared from PMA and ionomycin-stimulated mouse spleen cells (Fig. 2B). Similar to the human isoform, the additional band found in mouse contains the intron 4 sequences (i4) between the exons 4 and 5 of mIL-21 gene (Fig. 2A and D). This additional sequence in mIL-21iso also has an in-frame stop codon. The predicted mIL-21iso precursor protein contains 162 amino acids and mature mIL-21iso consists of 138 amino acids that is 16 amino acids longer than that of mIL-21. We confirmed that the mIL-21iso transcript was only found in activated spleen cells using mIL-21iso specific primer set (mF2 and mR2) (Fig. 2C). The sequences of hIL-21iso and mIL-21iso reported in this paper have been deposited in GenBank Accession Nos. DQ645417 and DQ645418, respectively.

Next we tried to detect IL-21 and IL-21iso protein by Western blotting but we could not detect both of them (data not shown). The amount of these proteins produced from activated $CD4^+$T cells might be less than the detection level.

3.2. Kinetics of IL-21 and IL-21iso mRNA expression

First, we compared the kinetics of IL-21 and IL-21iso mRNA expression in activated human $CD4^+$ T cells or mouse spleen cells by RT-PCR. Both human and mouse IL-21 and IL-21iso mRNA expressions were detected within 1 h after PMA and ionomycin stimulation and then upregulated, though a little transcript of IL-21 but not of IL-21iso was detected before stimulation (Fig. 3A, C and E). IL-21 and IL-21iso mRNA expressions persisted even at 48 h after stimulation (data not shown). As an internal control, we examined the level of β-actin mRNA. Although the expression patterns of IL-21 and IL-21iso were similar, the real time quantitative PCR experiments showed that the mRNA expression level of hIL-21iso was much lower than that of hIL-21 (Fig. 3C).

Cycloheximide treatment of human $CD4^+$ T cells or mouse spleen cells, which was conducted 1 h before the PMA and ionomycin stimulation, initially inhibited the expression of IL-21 mRNA partially but later on enhanced it, whereas cycloheximide inhibited mRNA expression of hIL-21iso moderately and significantly of mIL-21iso (Fig. 3A, C and E). These results indicate that IL-21iso mRNA expression depends on in part *de novo* protein synthesis. The cycloheximide induced-slight upregulation of IL-21 mRNA after activation was reported previously [25], and was similar to that of IL-2 mRNA as Zubiaga et al. reported [26]. These results suggest that there are different regulatory mechanisms between IL-21, IL-2 and IL-21iso mRNA expression.

Kim et al. reported that mIL-21 gene is activated with only ionomycin treatment [25]. We also observed that ionomycin treatment alone upregulated both IL-21 and IL-21iso mRNA expressions in human and mouse, whereas PMA alone could not (Fig. 3B, D and F). Because primers, which Kim et al. used to determine the mIL-21 mRNA levels, are on the 3′-UTR that is common to both mIL-21 and mIL-21iso transcripts, ionomycin-induced activation of mIL-21 gene in their study may reflect the up-regulation of both mIL-21 and mIL-21iso mRNA.

So far many cytokine isoforms have been reported. Even among the γc-dependent cytokines, IL-2, IL-4, IL-7 and IL-15 are known to have isoforms [20,21,27–29]. However, the control mechanisms for the isoform transcripts are largely unknown. We speculate that certain splicing negative regulatory molecules may be produced in these cells. Without these molecules due to the treatment of cycloheximide, intron 4 of premature IL-21 mRNA is spliced out and only mature IL-21 mRNA is prepared, however, in the presence of these molecules the splicing is partially inhibited and IL-21iso mRNA may be produced. Nucleotide sequences around 5′ splice site

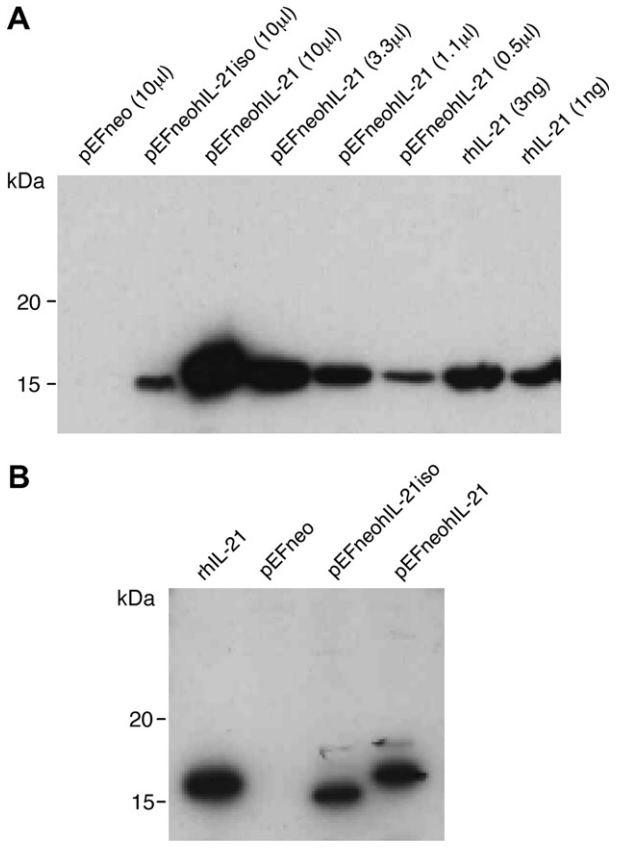

Fig. 4. Production of hIL-21 and hIL-21iso. (A) Cytokines in the supernatants of 293T cells transfected with pEFneo (empty vector), pEFneohIL-21iso or pEFneohIL-21 were detected by immunoblot assay using affinity purified anti-hIL21 polyclonal Ab. rhIL-21 were used as positive controls. The amounts of loaded supernatants are indicated. (B) Intracellular cytokines expressed in 293T cells transfected with pEFneo (empty vector), pEFneohIL-21iso or pEFneohIL-21 were detected by immunoblot assay using anti-hIL21 polyclonal Ab. 10 μg of each total cell extracts were used for assay. 10 ng rhIL-21 was used as a positive control.

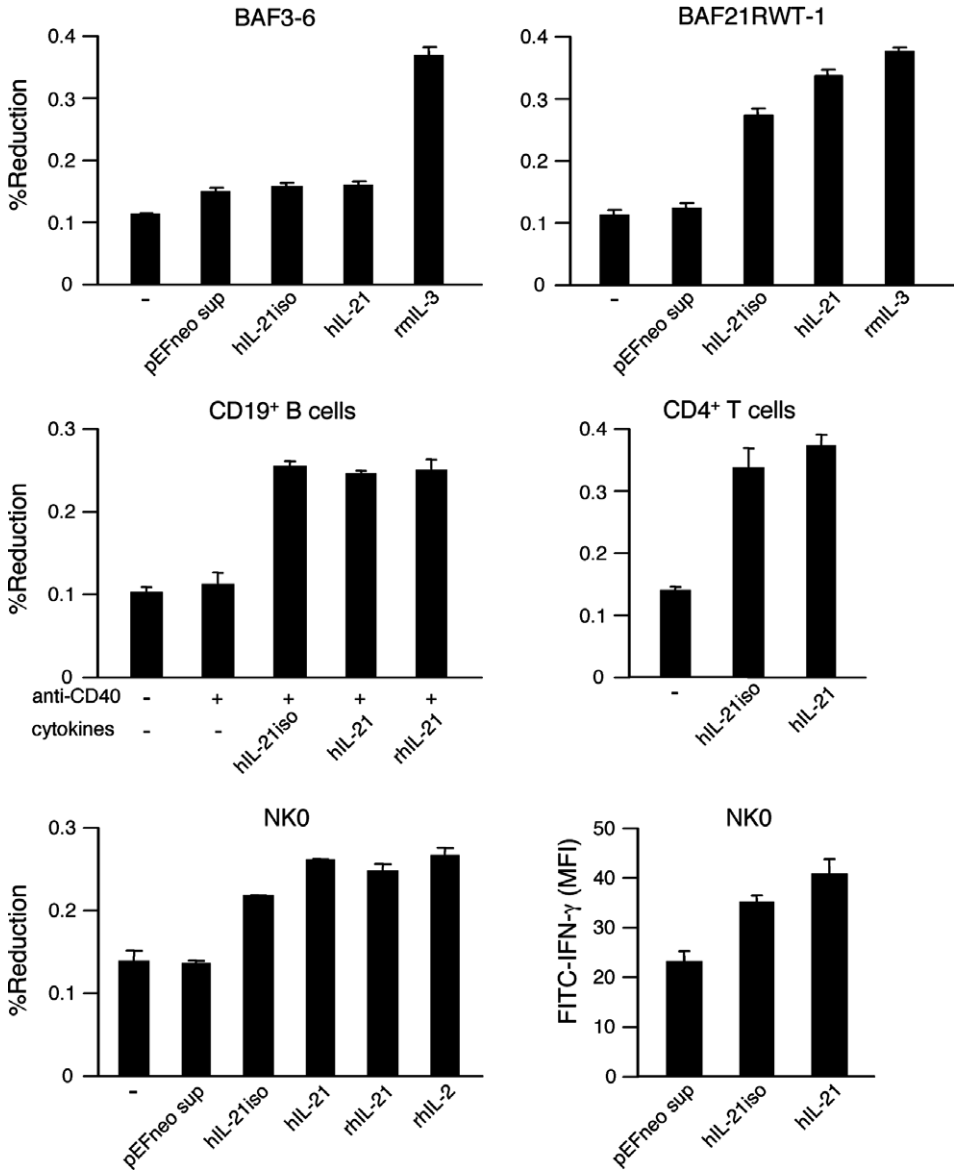

Fig. 5. Effect of IL-21 and IL-21iso on cell proliferation and IFN-γ production. Cell culture conditions, cell proliferation assay and IFN-γ production assay were described in Section 2. BAF3-6 and BAF21RWT-1 were stimulated with empty vector-transfected 293T cell supernatant, hIL-21iso, hIL-21 or rmIL-3 as indicated. $CD19^+$ B cells were stimulated with or without anti-CD40, and hIL-21iso, hIL-21 or rhIL-21 as indicated. $CD4^+$ T cells were cultured on anti-CD3 pre-coated plate and stimulated with hIL-21iso or hIL-21 as indicated. NK0 cells were stimulated with empty vector-transfected 293T cell supernatant, hIL-21iso, hIL-21, rhIL-21 or rhIL-2 as indicated. Mean fluorescence intensity (MFI) are shown for IFN-γ production assay. We stimulated cells with 25 ng/ml hIL-21iso, hIL-21 or rhIL-21, 1 ng/ml rmIL-3 or 1 nM rhIL-2 for each assay. Representative data are shown from three independent experiments.

and 3′splice site of intron 4 is conserved in human and mouse. However, several studies have shown that exonic and intronic sequences in additional to the well-conserved donor and acceptor sites may play a role in nuclear pre-mRNA splicing [30]. A genome wide survey shows that intron retention event (as in the case of IL-21iso) is usually found in genes with shorter introns (less than 200bp) but it is rarer than any other forms of alternative splicing [31]. Interestingly, dramatically higher frequency of exonic splicing silencers or depletion of exonic splicing enhancers is found in intron retention both in human and mouse [31]. Further studies are required to reveal the presence of any silencer or enhancer elements in IL-21 gene and some regulatory molecules involving in the splicing events.

3.3. Secretion of hIL-21iso

To produce hIL-21 and hIL-21iso, we transfected 293T cells with pEFneohIL-21 or pEFneohIL-21iso and the supernatants were subjected to SDS–PAGE and immunoblot assay using anti-hIL-21 mAb. We detected a single band of hIL-21iso with a little smaller size of molecular mass compared to a 293T cell expressed-hIL-21 and a control recombinant hIL-21 (Fig. 4A). Transfection efficiency of pEFneohIL-21 and pEFneohIL-21iso or produced amount of each protein in these cells had no differences (Fig. 4B and data not shown). However, secreted amount of hIL-21iso was approximately 20-fold lower than that of hIL-21 (Fig. 4A). Similarly, reduced secretion of hIL-21iso was also found in COS7 and

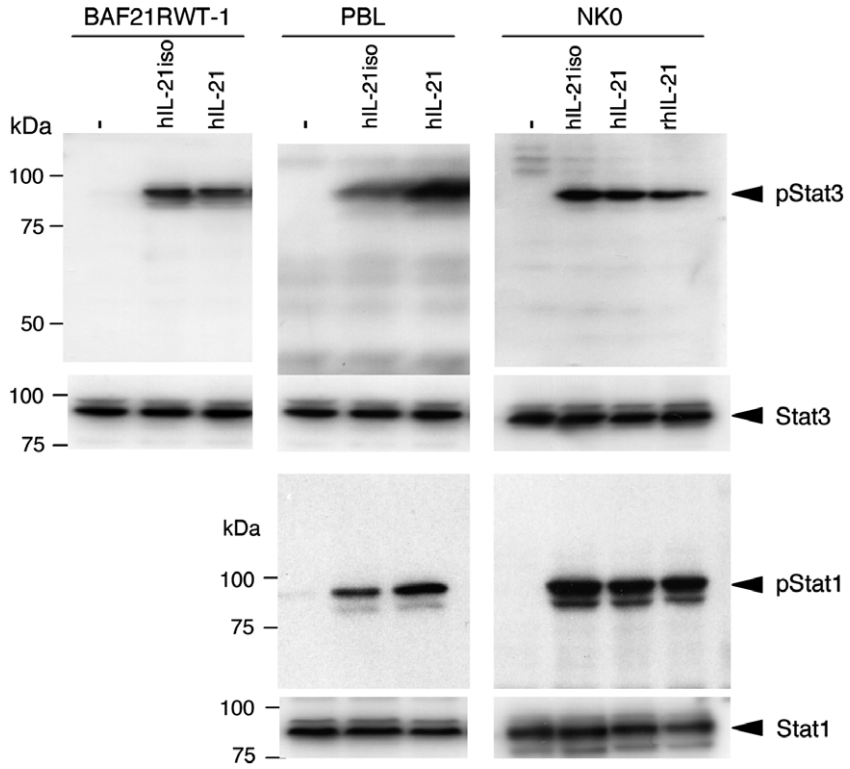

Fig. 6. STATs activation by hIL-21 or hIL-21iso. Cells were stimulated with hIL-21iso, hIL-21 or rhIL-21 as indicated for 30 min. Tyrosine-phosphorylated STATs were detected with anti-phospho STAT3 (pY705) or anti-phospho STAT1 (pY701) Abs. Membranes were reprobed with anti-STAT3 or anti-STAT1 Abs to confirm the protein levels. Protein molecular weight marker and the positions of each STAT are indicated.

HeLa cells transfected with pEFneohIL-21iso or pEFneohIL-21 (data not shown). Transcripts of IL-15 isoforms are known to have different translational efficiencies [29]. In our expression system, we could not find the different translational efficiencies between hIL-21 and hIL-21iso. The C-terminal differences between hIL-21 and hIL-21iso must be involved in the control of the secretory efficiency. hIL-21iso might work in the cells regulating cytokine signaling because produced hIL-21iso mainly remains in the cytoplasm and hIL-21-producing $CD4^+$T cells express both hIL-21 receptor and γc (data not shown).

3.4. Functional assay

Parrish-Novak et al. has already reported that hIL-21 induced proliferation of BaF3 cell line expressing IL-21R [1]. To check the function of hIL-21iso, we prepared hIL-21R-expressing BaF3 subline, BAF21RWT-1. hIL-21iso induced cell proliferation of BAF21RWT-1 but not of BAF3-6 parent cells and the supernatant from empty vector-transfected 293T cells had no functions on both cell lines (Fig. 5). Taken together, these results show that hIL-21iso transduces cell proliferation signals through a cognate receptor, IL-21R. hIL-21 costimulates proliferation of anti-CD40 Ab-activated B cells [1]. Similar to hIL-21, hIL-21iso costimulated proliferation of anti-CD40 mAb-induced $CD19^+$ B cells (Fig. 5). Also, we did not find any significant differences between the effect of hIL-21iso and hIL-21 on the proliferation of anti-CD3 mAb-activated $CD4^+$ T cells and on NK0 cells (Fig. 5). IL-21 is known to induce IFN-γ synthesis from human NK cells [32]. IFN-γ production from NK0 cells was increased after stimulation with hIL-21iso and hIL-21 (Fig. 5).

3.5. IL-21iso induced activation of STATs

IL-21 induces phosphorylation of STAT1 and STAT3 [3]. In order to compare signaling ability of IL-21iso and IL-21, BAF21RWT-1 cells, PBMC and NK0 cells were stimulated with either hIL-21iso or hIL-21. These cell extracts were subjected on SDS–PAGE and immunoblot assay with Abs specific for tyrosine phosphorylated STAT1 and STAT3. hIL-21iso induced tyrosine phosphorylation of STAT1 and STAT3 as well as hIL-21 in BAF21RWT-1 cells and NK0 cells while a little weakly in PBMC (Fig. 6). Mutations Q145D, I148D, and I148Stop within helix D of IL-21 were reported to reduce signaling capability but not to affect the receptor-binding characteristics [33]. Although the C-terminal 7 amino acids (amino acid Nos. 147–153) of hIL-21iso are different from hIL-21, signal transduction ability of hIL-21iso on BAF21RWT-1 and NK0 cells is quite similar to that of hIL-21. Since IL-21 has very diverse functions on various immune cells, more research based on this primary findings are needed to understand the physiological function of this novel IL-21 isoform.

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References

[1] Parrish-Novak, J., Dillon, S.R., Nelson, A., Hammond, A., Sprecher, C., Gross, J.A., Johnston, J., Madden, K., Xu, W., West, J., Schrader, S., Burkhead, S., Heipel, M., Brandt, C.,

Kuijper, C.H., Kramer, J., Conklin, D., Presnell, S.R., Berry, J., Shiota, F., Bort, S., Hambly, K., Mudri, S., Clegg, C., Moore, M., Grant, F.J., Lofton-Day, C., Gilbert, T., Raymond, F., Ching, A., Yao, L., Smith, D., Webster, P., Whitmore, T., Maurer, M., Kaushansky, K., Holly, R.D. and Foster, D. (2002) Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature 408, 57–63.

[2] Ozaki, K., Kikly, K., Michalovich, D., Young, P.R. and Leonard, W.J. (2000) Cloning of a type I cytokine receptor most related to the IL-2 receptor β chain. Proc. Natl. Acad. Sci. USA 97, 11439–11444.

[3] Asao, H., Okuyama, C., Kumaki, S., Ishii, N., Tsuchiya, S., Foster, D. and Sugamura, K. (2001) The common γ-chain is an indispensable subunit of the IL-21 receptor complex. J. Immunol. 167, 1–5.

[4] Habib, T., Weinberg, K. and Kaushansky, K. (2002) The common γ chain (γc) is a required signaling component of the IL-21 receptor and supports IL-21-induced cell proliferation via JAK3. Biochemistry 41, 8725–8731.

[5] Noguchi, M., Yi, H., Rosenblatt, H.M., Filipovich, A.H., Adelstein, S., Modi, W.S., McBride, O.W. and Leonard, W.J. (1993) Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. Cell 73, 147–157.

[6] Ozaki, K., Spolski, R., Feng, C.G., Qi, C.F., Cheng, J., Sher, A., Morse 3rd, H.C., Liu, C., Schwartzberg, P.L. and Leonard, W.J. (2002) A critical role for IL-21 in regulating immunoglobulin production. Science 298, 1630–1634.

[7] Jin, H., Carrio, R., Yu, A. and Malek, T.R. (2004) Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis. J. Immunol. 173, 657–665.

[8] Distler, J.H., Jungel, A., Kowal-Bielecka, O., Michel, B.A., Gay, R.E., Sprott, H. and Matucci-Cerinic, M. (2005) Expression of interleukin-21 receptor in epidermis from patients with systemic sclerosis. Arthritis Rheum. 52, 856–864.

[9] Kasaian, M.T., Whitters, M.J., Carter, L.L., Lowe, L.D., Jussif, J.M., Deng, B., Johnson, K.A., Witek, J.S., Senices, M., Konz, R.F., Wurster, A.L., Donaldson, D.D., Collins, M., Young, D.A. and Grusby, M.J. (2002) IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. Immunity 16, 559–569.

[10] Ozaki, K., Spolski, R., Ettinger, R., Kim, H.P., Wang, G., Qi, C.F., Hwu, P., Shaffer, P.J., Akilesh, S., Roopenian, D.C., Morse, H.C., Lipsky, P.E. and Leonard, W.J. (2004) Regulation of B Cell Differentiation and Plasma Cell Generation by IL-21, a Novel Inducer of Blimp-1 and Bcl-6. J. Immunol. 173, 5361–5371.

[11] Suto, A., Nakajima, H., Hirose, K., Suzuki, K., Kagami, S., Seto, Y., Hoshimoto, A., Saito, Y., Foster, D.C. and Iwamoto, I. (2002) Interleukin 21 prevents antigen-induced IgE production by inhibiting germ line Cε transcription of IL-4-stimulated B cells. Blood 100, 4565–4573.

[12] Harada, M., Magara-Koyanagi, K., Watarai, H., Nagata, Y., Ishii, Y., Kojo, S., Horiguchi, S., Okamoto, Y., Nakayama, T., Suzuki, N., Yeh, W.C., Akira, S., Kitamura, H., Ohara, O., Seino, K. and Taniguchi, M. (2006) IL-21-induced Bε cell apoptosis mediated by natural killer T cells suppresses IgE responses. J. Exp. Med. 203, 2929–2937.

[13] Toomy, J.A., Gays, F., Foster, D. and Brooks, C.G. (2003) Cytokine requirements for the growth and development of mouse NK cells in vitro. J. Leukocyte Biol. 74, 233–242.

[14] Sivori, S., Cantoni, C., Parolini, S., Marcenaro, E., Conte, R., Moretta, L. and Moretta, A. (2003) IL-21 induces both rapid maturation of human $CD34^+$ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors. Eur. J. Immunol. 33, 3439–3447.

[15] Brandt, K., Bulfone-Paus, S., Foster, D.C. and Ruckert, R. (2003) Interleukin-21 inhibits dendritic cell activation and maturation. Blood 102, 4090–4098.

[16] Strengell, M., Lehtonen, A., Matikainen, S. and Julkunen, I. (2006) IL-21 enhances SOCS gene expression and inhibits LPS-induced cytokine production in human monocyte-derived dendritic cells. J. Leukocyte Biol. 79, 1279–1285.

[17] King, C., Ilic, A., Koelsch, K. and Sarvetnick, N. (2004) Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 177, 265–277.

[18] Vollmer, T.L., Liu, R., Price, M., Rhodes, S., Cava, L.A. and Shi, F.D. (2005) Differential effects of IL-21 during initiation and progression of autoimmunity against neuroantigen. J. Immunol. 174, 2696–2701.

[19] Leonard, W.J. and Spolski, R. (2005) Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. Nat. Rev. Immunol. 5, 688–698.

[20] Tsytsikov, V.N., Yurovsky, V.V., Atamas, S.P., Alms, W.J. and White, B. (1996) Identification and characterization of two alternative splice variants of human interleukin-2. J. Biol. Chem. 271, 23055–23060.

[21] Atamas, S.P., Choi, J., Yurovsky, V.V. and White, B. (1996) An alternative splice variant of human IL-4, IL-4 delta 2, inhibits IL-4-stimulated T cell proliferation. J. Immunol. 156, 435–441.

[22] Lee, S.J., Cho, Y.S., Cho, M.C., Shim, J.H., Lee, K.A., Ko, K.K., Choe, Y.K., Park, S.N., Hoshino, T. and Yoo, D.Y. (2001) Both E6 and E7 oncoproteins of human papillomavirus 16 inhibit IL-18-induced IFN-γ production in human peripheral blood mononuclear and NK cells. J. Immunol. 167, 497–504.

[23] Asao, H. and Fu, X.Y. (2000) Interferon-gamma has dual potentials in inhibiting or promoting cell proliferation. J. Biol. Chem. 275, 867–874.

[24] Jordan, M. and Wurm, F. (2004) Transfection of adherent and suspended cells by calcium phosphate. Methods 33, 136–143.

[25] Kim, H.P., Korn, L.L., Gamero, A.M. and Leonard, W.J. (2005) Calcium-dependent activation of interleukin-21 gene expression in T cells. J. Biol. Chem. 280, 25291–25297.

[26] Zubiaga, A.M., Munoz, E. and Huber, B.T. (1991) Superinduction of IL-2 gene transcription in the presence of cycloheximide. J. Immunol. 146, 3857–3863.

[27] Korte, A., Moricke, A., Beyermann, B., Kochling, J., Taube, T., Kebelmann-Betzing, C., Henze, G. and Seeger, K. (1999) Extensive alternative splicing of interleukin-7 in malignant hematopoietic cells: implication of distinct isoforms in modulating IL-7 activity. J. Interferon Cytokine Res. 19, 495–503.

[28] Meazza, R., Verdiani, S., Biassoni, R., Coppolecchia, M., Gaggero, A., Orengo, A.M., Colombo, M.P., Azzarone, B. and Ferrini, S. (1996) Identification of a novel interleukin-15 (IL-15) transcript isoform generated by alternative splicing in human small cell lung cancer cell lines. Oncogene 12, 2187–2192.

[29] Nishimura, H., Washizu, J., Nakamura, N., Enomoto, A. and Yoshikai, Y. (1998) Translational efficiency is up-regulated by alternative exon in murine IL-15 mRNA. J. Immunol. 160, 936–942.

[30] Black, D.L. (2003) Mechanisms of alternative pre-messenger RNA splicing. Annu. Rev. Biochem. 72, 291–336.

[31] Zheng, C.L., Fu, X.D. and Gribskov, M. (2005) Characteristics and regulatory elements defining constitutive splicing and different modes of alternative splicing in human and mouse. RNA 11, 1777–1787.

[32] Strengell, M., Sareneva, T., Foster, D., Julkunen, I. and Matikainen, S. (2002) IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. J. Immunol. 169, 3600–3605.

[33] Parrish-Novak, J., Foster, D.C., Holly, R.D. and Clegg, C.H. (2002) Interleukin-21 and the IL-21 receptor: novel effectors of NK and T cell responses. J. Leukocyte Biol. 72, 856–863.